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14. ABSTRACT Magnetic Resonance Imaging is an emerging diagnostic tool for breast cancer that provides high-resolution three-dimensional images of tissues. A significant increase of specificity of detection of cancerous lesions could be achieved by use of tumor specific contrast agents (CA). Currently, tumor-specific targeting of CA is achieved by antibodies (Ab) or fragments thereof. The major disadvantages of Ab are their large size and ability to induce immune response. We proposed to use DNA aptamers for tumor-specific targeting of CA because short DNA molecules are smaller than Ab, have better tissue penetration and are non-toxic and non-immunogenic. During the study, PSI developed an experimental system and generated essential reagents for selection of DNA aptamers. Expression constructs encoding human EGF receptor (EGF-R) fusion protein were produced and used for generation of stable cell lines expressing this protein at suitable levels. EGF-R was used for isolation of DNA aptamers from combinatorial synthetic library. A group of structurally related aptamers was identified. In the course of the program a variety of technical challenges were identified and alternative approaches were undertaken. Full characterization and validation of the isolated EGF-R-specific aptamers was not completed. PSI research effort generated valuable reagents and technical knowledge for further development of the proposed technology.

15. SUBJECT TERMS
Magnetic Resonance Imaging, MRI of Breast, Aptamer, Oligonucleotide Library, Contrast Agent, HER2, EGF Receptor, Nanoparticles, Metastasis Detection, Breast Tumor

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INTRODUCTION:

Magnetic Resonance Imaging (MRI) is an emerging diagnostic tool for breast cancer management [1]. This extremely powerful technique provides a high-resolution three-dimensional image portraying morphological features of tissues and organs [2]. However, the anatomic details delineated even at very high resolution are not sufficient for diagnosis of malignant lesions as they are often indistinguishable from normal and benign structures. Increase in specificity of detection of cancerous lesions is currently achieved by use of intravenous contrast agents. The current approach for targeting of tumor-specific contrast agents is based on conjugation of non-specific contrast agents (such as Gd chelates or mono- or polycrystalline iron oxide) with antibodies or fragments thereof [2]. Several successful attempts of delivering contrast agents to certain tumors have been reported (rev. in [2]) including targeting of Gd-based contrast agents to breast tumors expressing HER2/Neu receptor [3]. The major disadvantages of antibody-mediated targeting of contrast agents are large size of antibody molecules and their ability to induce undesirable immune response in a patient. An alternative approach for contrast agents targeting could be utilization of aptamers, synthetic single-stranded DNA molecules that bind to target molecules with high affinity and specificity [4]. Aptamers are much smaller than Abs yet display comparable binding properties. Aptamers used for animal studies so far were found to be non-toxic and non-immunogenic, features important for use in humans. Application of aptamers as targeting moieties for contrast agents has not been reported yet thus warranting research efforts in this direction.

BODY:

The objective of the program was to test our novel concept of using specific DNA aptamers for targeting MRI contrast agent to the surface of breast cancer cells.

The rationale of the proposed research effort was that targeting of contrast agents to tumor cells expressing surface markers is an efficient way to improve specificity and sensitivity of MRI of breast cancer lesions. Aptamers have significant advantages over antibodies such as small size and lack of immunogenisity and toxicity. **The novelty of our concept is the application of aptamers for targeting MRI contrast agents for detection of breast tumors.** MRI sensitivity allows detection of a single cell in the whole body imaging which makes this technique useful for detection of small metastatic lesions and even individual tumor cells.

The design of the research included several consecutive steps including generation of expression DNA construct for production of isoform Erb-B2 of human HER2/Neu, , mutant protein, purification of the mutant, isolation of Erb-B2-specific aptamers and their characterization. Finally, we proposed to use isolated Erb-B2 aptamers for cell culture experiments to test their targeting properties. In the course of the study, we have performed the following experiments and obtained results as presented below.

Results

Design of experimental approach.

For selection of Erb-B2 specific aptamers, we proposed to utilize whole cells expressing cleavable Erb-B2 mutant. In this approach, combinatorial synthetic aptamer library is incubated with whole cells and aptamers specific to Erb-B2 bind to the extracellular part of the molecule which is then specifically cleaved off of the cells by highly selective Tobacco Etch Virus (TEV) protease (Figure 1). This approach was thought to provide higher selection pressure than use of purified protein preparations because aptamers that bind to other cellular proteins are removed from competition for binding sites on Erb-B2.

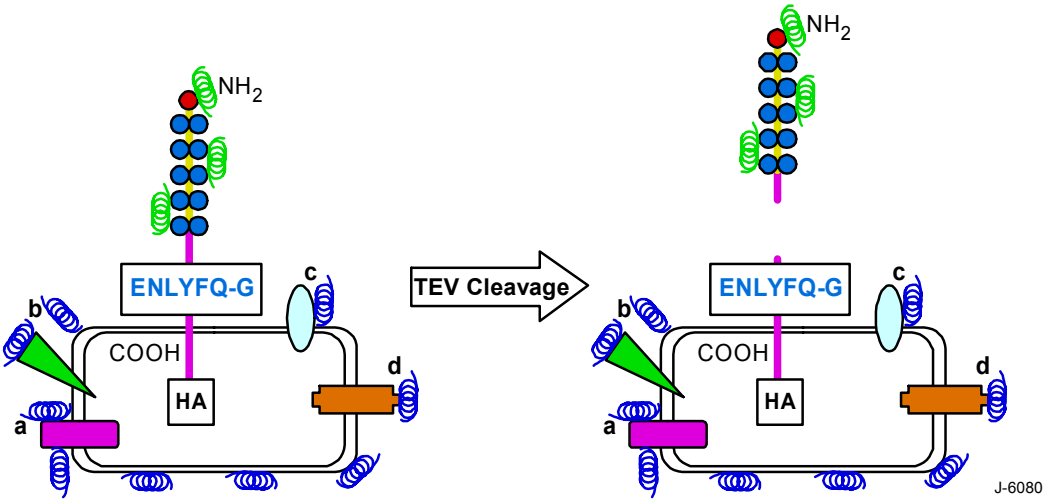

Figure 1. Schematic diagram of the originally proposed aptamer isolation procedure. Extracellular portion of human Erb-B2 was left unmodified with the exception of introduction of TEV cleavage site (ENLYFQ-G) juxtaposed to transmembrane domain. Cytoplasmic fragment of Erb-B2 was truncated and replaced with HA epitope tag. TEV mediated cleavage (arrow) releases the extracellular fragment of the molecule along with aptamers bound to it (green spirals). Aptamers capable of binding to other cellular proteins (blue spirals) are sequestered from the library resulting in increase of specificity of aptamer selection process.

Generation of DNA constructs for expression of His-tagged HER2/neu fusion proteins.

To produce protein target suitable for aptamer selection procedure, we generated human Erb-B2 epitope tagged constructs (Figure 2). N-terminal extracellular fragment of Erb-B2 molecule was kept intact with the addition of 6-amino acid cleavage site for TEV protease positioned ~30 amino acids from the transmembrane region. Two alternative constructs harboring HA- and V5-epitope tags were also generated. Since we proposed to generate stable cell lines expressing Erb-B2 mutants, in order to facilitate selection of positive clones we genetically attached Green Fluorescent Protein (GFP) to the intracellular part of Erb-B2 molecule. Addition of GFP to the construct provides a means of identifying cells with good expression of the fusion protein. HA- and V5-tagged Erb-B2 fragments were cloned into pIRES

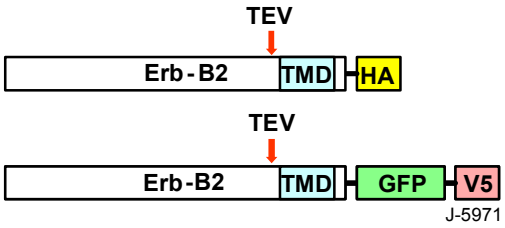

Figure 2. Schematic design of Erb-B2 expression constructs. N-terminal extracellular and transmembrane (TMD) domains of Erb-B2 were identical to the native protein with the exception of TEV protease cleavage site introduced 30 amino acid residues upstream of TMD. The cytoplasmic fragment of Erb-B2 was truncated and replaced either with HA-epitope tag or with GFP followed by V5 tag. Red arrow indicates approximate location of the TEV cleavage site.

Puro 3 (Clontech) and pEF6-6His-V5 (Invitrogen) mammalian expression vectors, respectively. Both constructs contain 6-His purification tag located immediately upstream of both HA- and V5 epitope tags (not shown on the figure). Each construct was generated with or without TEV cleavage site to demonstrate specificity of TEV-mediated cleavage at later stages of the research project. All constructs were verified by sequencing analysis prior to transfection experiments.

Transient expression of recombinant Erb-B2

To test efficiency of protein expression from the generated DNA constructs, we started with Erb-B2 constructs containing GFP and V5 tags as those were expected to display green fluorescence for simplification of stable cell line generation. Both constructs, Erb-B2-GFP-V5 and Erb-B2-TEV-GFP-V5, were transiently transfected into U2-OS cell line and expression of the recombinant proteins was detected by Western blot (Figure 3). In the same experiment, HA-tagged Erb-B2 mutants were also tested. Expression of all four mutants was readily detected by Western blot suggesting suitability of the generated expression constructs for further experiments.

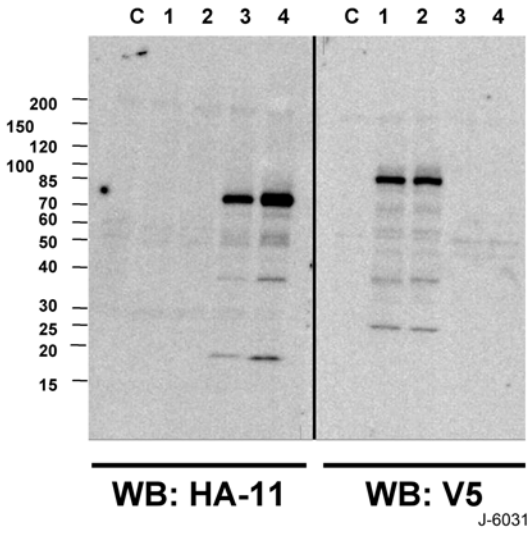

Figure 3. Transient transfection of various Erb-B2 mutants. Whole cell extracts (20 μg per lane) were prepared from U2-OS cells transfected with Erb-B2-GFP-V5 (Lane 1), Erb-B2-TEV-GFP-V5 (Lane 2), Erb-B2-HA (Lane 3) and Erb-B2-TEV-HA. Lane C represents a control extract prepared from non-transfected cells. The samples were stained with anti-HA (left panel) and anti-V5 (right panel) antibodies. Protein markers (in kDa) are shown on the left.

Generation of stable cell lines expressing recombinant Erb-B2

For generation of the stable cell lines expressing Erb-B2 fusion proteins, U2-OS cell were transfected and then 2 days later split into selection medium containing blasticidin. At this point, we attempted to visualize GFP fluorescence but the signal was undetectable. Lack of detectable fluorescence could be attributed to the lack of proper folding of GFP fragment due to proximity of transmembrane domain and membrane anchoring. Individual colonies formed after 7 – 8 days of culture propagation of the selection medium were randomly picked up and transferred in 24-well plates. After another 3 days, the wells were harvested for further propagation and for Western blot analysis using anti-V5 antibodies. We have analyzed whole cell extracts from 6 independent clones each transfected with Erb-B2-GFP-V5 or Erb-B2-TEV-GFP-V5. As shown on Figure 4, clones #1 and #5 from Erb-B2-GFP-V5 and clone #4 from Erb-B2-TEV-GFP-V5 cell lines displayed the highest level of expression of the recombinant proteins. Migration patterns of both proteins were consistent with anticipated molecules mass of the proteins (~105 kDa). The clones displaying highest protein expression were selected, expanded and cryopreserved for further use.

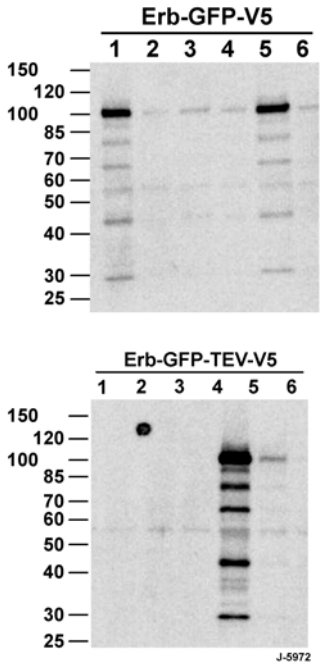

Figure 4. A representative Western blot analysis of recombinant protein expression in individual clones of U2-OS cells. Whole cell extracts for each indicated construct were loaded on the gel (20 μg per lane) and probed with anti-V5 antibodies. Protein markers (in kDa) shown on the left.

Characterization of stable cell lines expressing Erb-B2 mutants by immunofluorescence

To evaluate homogeneity of protein expression within the selected clones, we carried out immunofluorescence studies. Individual U2-OS clones expressing Erb-B2-GFP-V5 or Erb-B2-TEV-GFP-V5 were grown on coverslips, fixated, permeabilized and stained with anti-V5 antibodies. Figure 5 (Panel A and B) shows representative staining patterns for both constructs (Erb-B2-GFP-V5 and Erb-B2-TEV-GFP-V5 respectively) visualized by Cy3-labeled secondary antibodies. Merging DAPI staining for DNA with Cy3 demonstrated that >90% nucleus containing cells also expresses detectable amounts of a recombinant Erb-B2 protein. Importantly, level of expression essentially in all cells was comparable suggesting that these clones are suitable for protein production. Analysis of protein expression of the corresponding clones by Western blot is shown on Figure 5, C.

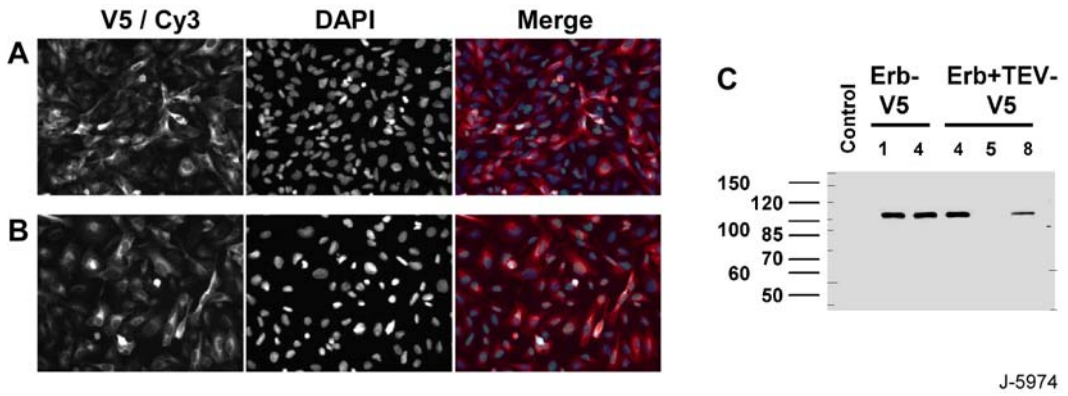

Figure 5. Immunofluorescence analysis of cell lines expressing Erb-B2 recombinant proteins. The cells expressing Erb-B2-GFP-V5 or Erb-B2-TEV-GFP-V5 mutants were propagated on coverslips, fixated, permeabilized and stained with DAPI (DAPI) to detect DNA and anti-V5 antibodies (V5/Cy3) for recombinant protein detection. Panel A shows staining of Erb-B2-GFP-V5, clone #4 and Panel B – Erb-B2-TEV-GFP-V5, clone #4. Western blot analysis of the same clones is shown on Panel C (please not three other clones and a control lane are included in this panel).

Demonstration of membrane association of Erb-B2 mutants expressed in stable cell lines

Erb-B2 mutant design relied on the assumption that membrane targeting and natural folding of the protein will be achieved when expressed in human cell. Therefore, it was important to test if the Erb-B2 mutants are targeted to the cell membrane. Immunofluorescence studies reveled that recombinant Erb-B2 was apparently associated with cell membrane, however, substantial amounts of this protein appeared to be detected in cytoplasm of the cells. To address membrane association of Erb-B2 mutants more carefully, we carried out cellular fractionation experiments and measured relative amount of V5-tagged mutant in different fractions. The cells expressing mutant Erb-B2 were resuspended in hypotonic buffer and ruptured using a Dounce homogenizer. The soluble cytoplasmic fraction was separated from cell debris by centrifugation and the resulting fractions were analyzed by Western blot (Figure 6). We found that both cleavable and non-cleavable Erb-B2 mutants are essentially exclusively associated with membrane fraction. Presence of minor amounts of Erb-B2 protein in cytoplasmic fraction of Erb-B2-TEV-GFP-V5-expressing cells could be attributed to partial contamination of this fraction by cell membranes (Figure 6). Additionally, we established that relative amount of Erb-B2 in membrane fraction is 2-3 fold higher than in whole cell extract, further indicating membrane association of Erb-B2 and its enrichment upon membrane isolation procedure.

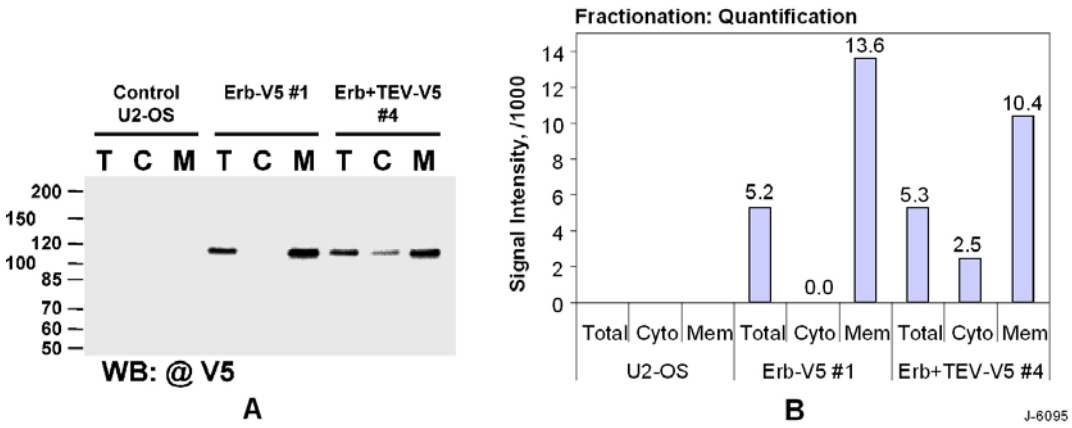

Figure 6. Fractionation of cells expressing Erb-B2 mutant protein. Parental U2-OS cells and their derivatives expressing Erb-B2-GFP-V5 or Erb-B2-TEV-GFP-V5 were fractionated and 20 μg of each fraction was loaded per lane. The cells were harvested and resuspended in hypotonic buffer to induce cell lysis. After lysis, cell membranes and debris were separated from the cytoplasmic fraction by centrifugation and the resulting fractions were analyzed by Western blot using anti-V5 antibodies (Panel A). Total cell lysate, cytoplasmic and membrane fractions are indicated by T, C and M respectively. Panel B: quantification of the Western blot shown on Panel A. Signal intensity was measured by FluorChem SP2 imaging station (Alpha Innotech).

TEV-mediated cleavage of Erb-B2 mutants expressed on surface of cells

After generation of a stable cell line expressing TEV cleavable mutant of Erb-B2, we tested the aptamer selection approach originally proposed in our grant application. Schematically this approach is represented on Figure 1 where extracellular portion of the Erb-B2 fusion protein expressed on the cell surface was planned to be removed by TEV mediated proteolysis. We anticipated that due to high specificity of TEV cleavage, purification of the protein would be unnecessary. To test this approach, stable cell lines expressing either cleavable or non-cleavable mutants of Erb-B2 were harvested, washed with PBS to remove contaminating proteins (from serum supplement and secreted proteins) and incubated with TEV protease (Figure 7, A). The supernatant was analyzed for released extracellular portion of Erb-B2 mutant by silver stain to detect proteins. We found no detectable generation of the expected cleavage product with anticipated molecular mass of 85 kDa (Figure 7, B). We also observed substantial contamination of the supernatants of all three tested cell lines with non-relevant proteins originated from serum supplement, proteins secreted by the cells or combination of both. Further, we analyzed the remaining cells to see if TEV cleavage took place under conditions of the experiment. We detected no cleavage either by reduction of the amount of the full length protein or by generation C-terminal clipped product (Figure 7, C).

From this experiment we concluded that cleavage of Erb-B2 mutant on the cell surface is extremely inefficient and contamination levels are not suitable for aptamer selection. Lack of cleavage could be attributed to spatial hindrance of TEV cleavage site due to structural properties of the Erb-B2 mutant or due to its interaction with other cellular proteins. Therefore, we have modified our original approach to reduce complexity of the system and to provide better purity of protein preparations.

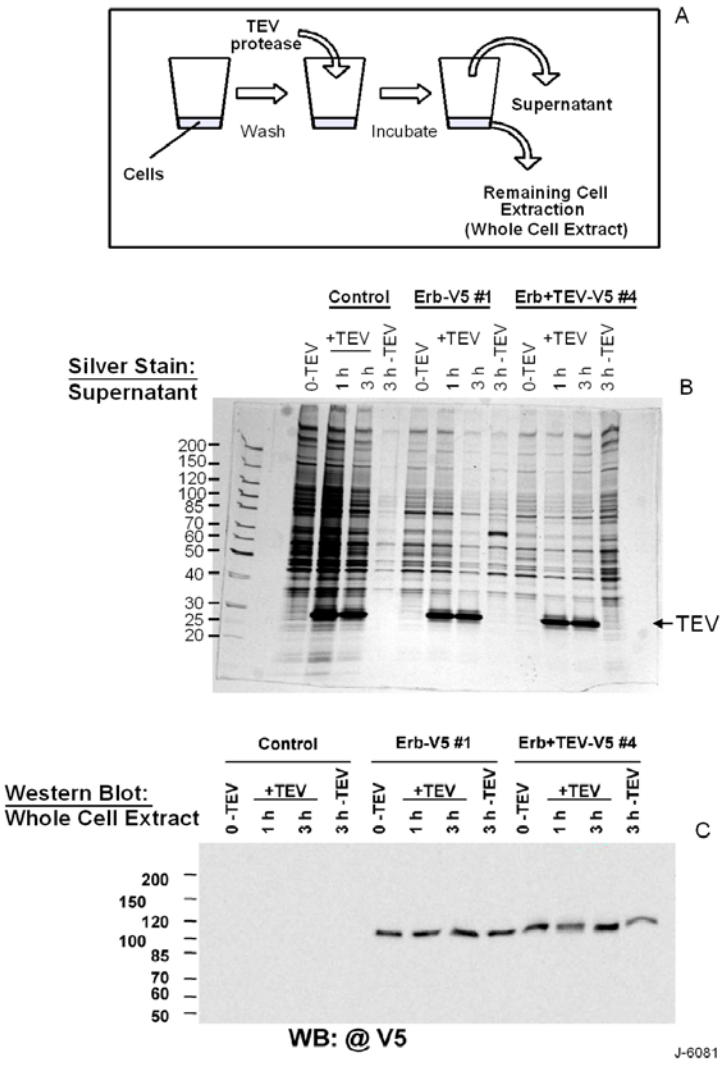

Figure 7. Cleavage of Erb-B2 mutant on the cell surface. Panel A: Schematic diagram of the experiment. Cells were extensively washed with PBS to remove contaminating soluble proteins and then incubated with TEV protease. After 1 and 3 hours of incubation, supernatant was removed and analyzed for release of extracellular portion of Erb-B2 (Supernatant). The remaining cells were extracted with SDS-PAGE loading buffer and analyzed for TEV mediated cleavage of Erb-B2 mutant (Whole Cell Extract). Panel B: Silver stain protein analysis of supernatant fractions obtained after incubation of parental 293 cells (Control) and cell lines expressing either non-cleavable (Erb-V5 #1) or cleavable (Erb+TEV-V5 #4) mutants. Protein markers are shown on the left and migration position of the added TEV protease is shown by the arrow on the right. Panel C Western blot analysis of whole cell extracts after incubation with TEV protease. Cell lines designated as described for Panel B.

TEV-mediated cleavage of Erb-B2 mutants in membrane preparations

As an alternative to the whole cell approach, we tested TEV mediate proteolysis on the plasma membranes isolated from the cells expressing Erb-B2 mutants, reasoning that membrane preparations could be less complex and heterogeneous than live cells. Additionally, cells always either secret or 'leak' proteins introducing contamination that cannot be controlled. First, we tested susceptibility of the expressed mutant proteins to the TEV-mediated proteolysis. Membranes were isolated from the stable cell lines expressing Erb-B2-TEV-GFP-V5 protein and either used directly for the TEV cleavage assay or were extracted with 0.5% Triton X-100 prior to the TEV proteolysis. The cleavage reaction was carried out for various times and the samples were analyzed by Western blot to detect degradation of the full length protein and generation of V5-tagged C-terminal product. As shown on Figure 8, both preparation of Erb-B2-TEV-GFP-V5 (membranes and extracts) were efficiently cleaved by TEV protease. As early as 30 min of incubation resulted in efficient degradation of considerable fraction (>80%) of the full length protein with concomitant formation of 40 kDa cleavage product.

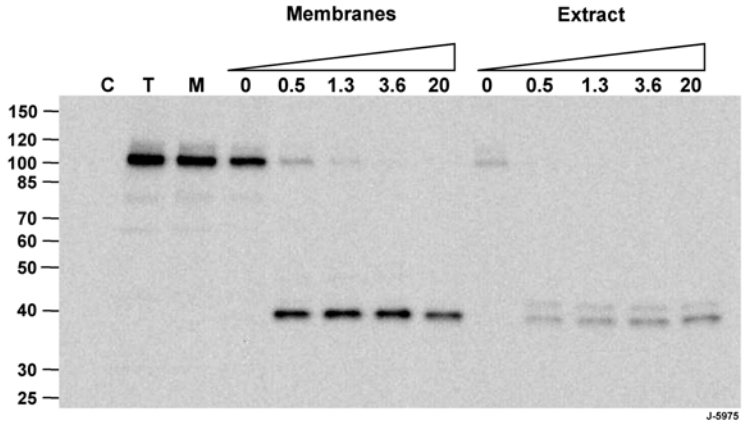

Figure 8. TEV protease cleavage of Erb-B2-TEV-GFP-V5 fusion protein. Suspension of cell membranes (Membranes) or their 0.5% Triton X-100 extract (Extract) were incubated with TEV protease for the indicated times (hours). The proteolysis was stopped by SDS-PAGE loading buffer containing 2% SDS and the resulting samples were loaded on the gel. Western blot was carried out using anti-V5 antibodies. Lane C is a control lane where membrane preparation of parental 293FT cells was loaded; T – is total extract of Erb-B2-TEV-GFP-V5-expressing cells and M – is the membrane fraction of these cells. Protein markers (in kDa) shown on the left.

We concluded that in both preparations, membranes and Triton X-100 extract, TEV cleavage site is accessible for the protease and could be used in protein purification procedure.

TEV cleavage of Erb-B2 mutants membrane preparations on filter membranes

To test suitability of on-membrane TEV cleavage for aptamer selection procedure, we isolated membrane fraction generated by rupture of Erb-B2 expressing cells in Dounce homogenizer in hypotonic buffer. The membranes then were placed on 0.2 μ filters, washed and subjected to TEV proteolysis as schematically presented on Figure 9, A and the resulting fraction

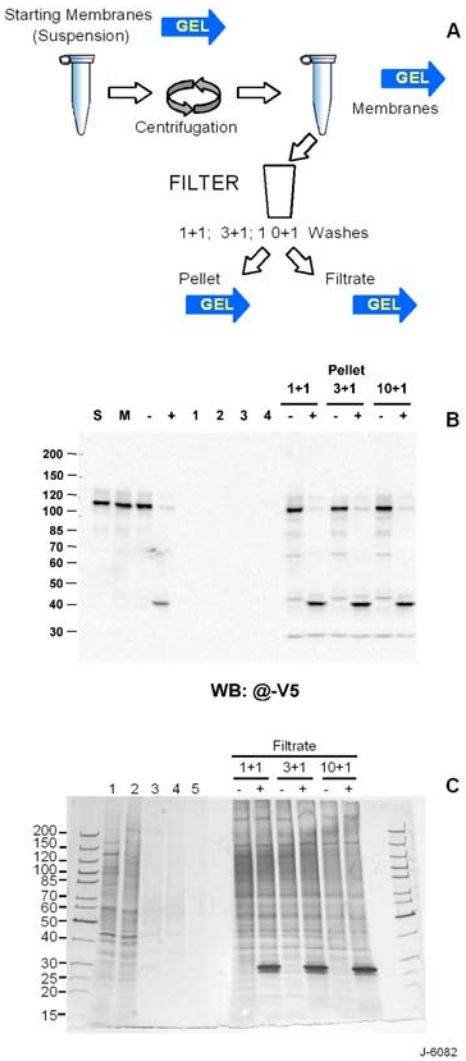

Figure 9. Cleavage of Erb-B2 mutant in membrane preparations. Panel A: Schematic diagram of the experiment. Cells were ruptured in hypotonic buffer and subjected to centrifugation. Pelleted membranes were resuspended, placed on 0.2 μ filters and washed by high salt washing buffer 1, 3 or 10 times. At the end of washes, the membranes were washed once with low salt buffer which was compatible with TEV cleavage reaction. Blue arrows indicated steps where SDS-PAGE samples were withdrawn for analysis. Panel B: Western blot analysis from the procedure described in Panel A. Lanes S and M represent original suspension of the cells and membrane fraction isolated by centrifugation, respectively. Lanes '-' and '+' indicated membrane preparation (M) incubated without or with TEV protease respectively. Lane 1 – cytoplasmic fraction obtained after centrifugation and pelleting plasma membranes; Lanes 2, 3 and 4 are the last washes after step 1+1, 3+1 and 10+1 prior to addition of TEV protease, respectively. The indicated washed pellets were treated with TEV protease (+) or left untreated (-). Panel C: Silver stain protein analysis of fractions passed through filters after incubation with TEV protease (+) or control incubation (-). Lane 1 – cytoplasmic fraction obtained after centrifugation and pelleting plasma membranes; Lane 2 – first wash of the filter after loading membrane preparations; Lanes 3, 4 and 5 are the last washed after step 1+1, 3+1 and 10+1 prior to addition of TEV protease, respectively.

were analyzed by Western blot and silver stain protein detection assay. Western blot analysis demonstrated that recombinant Erb-B2 preparation exposed to TEV protease on filters is a readily cleaved by the enzyme. This suggests that Ebr-B2 cleavage on filters could be potentially useful for isolation of aptamer/protein complexes. However, less encouraging results were revealed by silver staining of the corresponding filtrate fraction (Figure 9, C). Indeed, a substantial amount of protein contaminants was originating from the membrane preparations upon incubation with or without TEV protease and there was no detectable difference in the contamination pattern between membranes washed once, three or ten times.

This series of experiments demonstrated that cleavage of membrane-associated Erb-B2 mutants is unlikely to provide protein purity sufficient for aptamer selection process. If membrane preparation is incubated with synthetic oligonucleotide library and aptamer complexes are released by TEV cleavage on filters, a majority of such complexes will be presented by irrelevant proteins since target Erb-B2 complexes represent negligible fraction of total proteins eluted from the membranes (Figure 9, C).

Generation of stable cell lines with high expression levels of Erb-B2 mutants

We arrived to a conclusion that neither whole cell nor membrane preparation approach could be modified or optimized for selection of aptamers. Therefore, we considered adapting protein purification procedure for isolation of Erb-B2 along with its specific aptamers. Although expression of recombinant Erb-B2-GFP-V5 or Erb-B2-TEV-GFP-V5 was readily detectable by Western blot and cell staining, the amounts of protein produced by these cell lines was relatively low (data not shown). Low expression limited use of these cells for preparative protein production, therefore we used an alternative strategy for which we used different parental cell line and expression constructs (Figure 10).

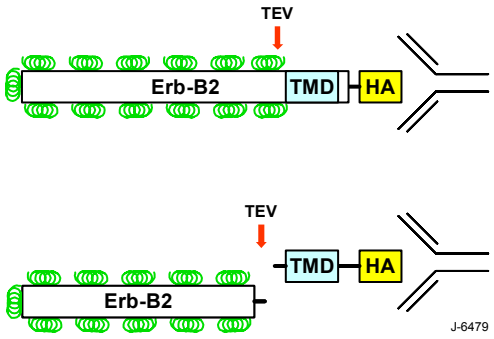

Figure 10. Schematic design of aptamer isolation approach. Erb-B2 fusion protein expressed in 293 HEK cells is incubated with aptamer library and then isolated by immunoprecipitation. Cleavage of the fusion protein by TEV protease will result in elution of the Erb-B2 portion of molecules from the beads along with aptamers bound to this fragment (green spirals).

293FT cell line (ATCC) was used as parental cell line because these cells express SV40 Large T antigen which significantly increases expression driven from viral promoters. Additionally, these cells have higher than U2-OS ratio of membrane-to-cytoplasm, a useful feature for production and isolation of membrane proteins. The coding sequences for the

expression constructs were subcloned into a vector with puromycin resistance which provides much more rapid and efficient selection than blasticidin. For this purpose we used pIRES-Puro3 vector harboring puromycin resistance and containing viral promoter compatible with 293FT cells. No intrinsic fluorescence of GFP of Erb-B2-GFP-V5 and Erb-B2-TEV-GFP-V5 constructs was detected (see above), hence we used Erb-B2-HA and Erb-B2-TEV-HA expression constructs (Figure 1) which encoded shorter (85 kDa) and more efficiently expressed recombinant proteins. Individual clones were selected by propagation of 293 cell transfected with the expression constructs in puromycin-containing medium. A representative analysis of individual clones is shown on Figure 11. Protein expression varies among different clones and the clones with highest expression were selected for further experiments.

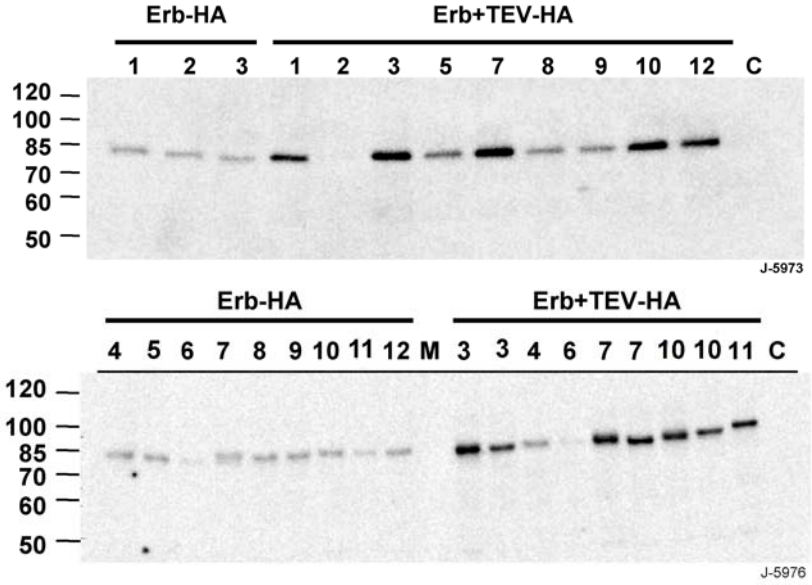

Figure 11. Western blot analysis of recombinant protein expression in individual clones of 293FT cells. Whole cell extracts for each indicated construct were loaded on the gel (20 μg per lane) and probed with anti-HA antibodies. Upper and lower panels represent two different experiments. Protein markers (in kDa) shown on the left.

TEV-mediated cleavage of Erb-B2 mutants in detergent extracts of membranes

Since we decided to modify our aptamer selection by using protein purification procedure, extraction of proteins from membranes was the first step. Solubilization of membrane proteins by detergents is known to induce alteration of protein conformation. We were concerned whether detergent extraction will render TEV cleavage site inaccessible for the protease. To test whether TEV cleavage site in Erb-B2-TEV-HA construct is accessible, a 0.5% Triton X-100 extract of membranes prepared from the cells expressing this protein was treated with TEV protease. As shown on Figure 12, detectable cleavage was observed at 15 min and by 2 hours more than 70% of initial amount of the full length protein was processed. Apparently low abundance of the 20 kDa clipped product could be attributed to a lower retention of this product due to reduced interaction with nitrocellulose.

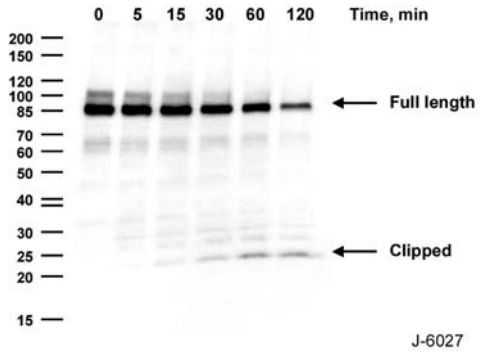

Figure 12. TEV protease cleavage of Erb-B2-TEV-HA fusion protein. Triton X-100 extract was incubated with TEV protease for the indicated times (min). The proteolysis was stopped by SDS-PAGE loading buffer containing 2% SDS and the resulting samples were loaded on the gel. Western blot was carried out using anti-HA antibodies. Protein markers (in kDa) shown on the left.

This experiment demonstrated that detergent extraction of cleavable Erb-B2 mutant retain accessibility of the TEV cleavage site for the protease. We concluded that solubilization of recombinant Erb-B2 protein is compatible with further protein purification procedure and TEV-mediated elution.

TEV-mediated cleavage of Erb-B2 mutants in immunoprecipitation reaction on beads

Use of TEV cleavage was anticipated to provide a specific and efficient way to elute proteins from immobilized antibodies. We tested whether the TEV cleavage also takes place when cleavable Erb-B2 fusion protein is bound to epitope-specific antibodies. Erb-B2-TEV-HA was extracted from cell membranes by 0.5% Triton X-100 and immunoprecipitated using anti-HA antibodies immobilized on agarose beads (Covance). The beads were extensively washed and subjected to TEV proteolysis. After incubation with TEV protease, SDS-PAGE loading buffer was added to the reactions and the resulting preparations were analyzed by silver stain and Western blot (Figure 13, A and B). As shown on Figure 13, B, antibody-bound form of Erb-B2-TEV-HA is readily cleaved by TEV protease demonstrating accessibility of the cleavage site under the experimental conditions. Silver staining revealed that Erb-B2 fusion protein could be detected in immunoprecipitation reactions as a protein band migrating close to 85 kDa protein marker. TEV cleavage results in conversion of this 85 kDa band into a shorter fragment migrating close to the 70 kDa protein marker and this reduction of molecular mass is consistent with removal of cytoplasmic fragment and epitope and purification tags (Figure 13, A, lane +TEV, panel IP).

This experiment demonstrated that TEV-mediated proteolysis takes place in Erb-B2 preparations bound to epitope-tag antibodies demonstrating accessibility of the cleavage site. However, it was also important to test whether extracellular domain of Erb-B2 dissociates from the antibody-bound proteins under non-denaturing conditions.

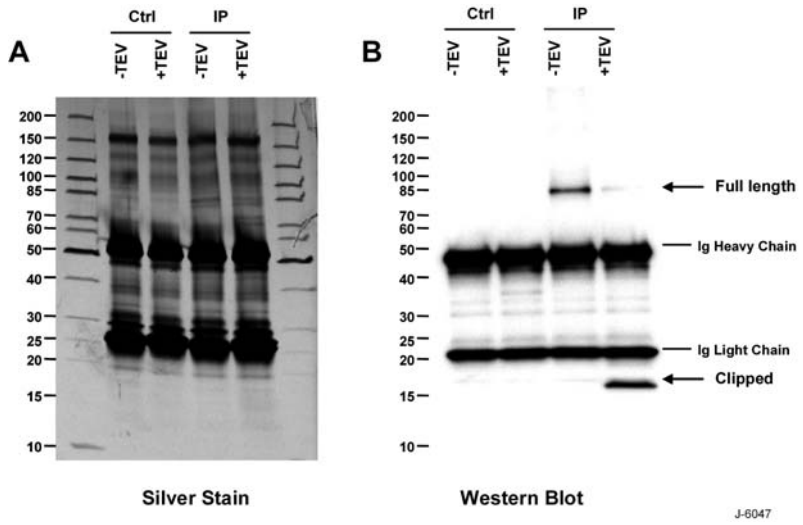

Figure 13. TEV cleavage of Erb-B2-TEV-HA in immunoprecipitation reactions. Triton X-100 extract of 293 cells stably expressing Erb-B2-TEV-HA (IP) and of parental 293 cell line (Ctrl) were incubated with anti-HA antibodies immobilized on agarose beads. Immunoprecipitation reaction was treated with TEV protease (+TEV) for 2 hours or left untreated (-TEV). Proteins were eluted from beads using SDS-PAGE loading buffer and analyzed by protein staining (Silver Stain, Panel A) and by Western blot using anti-HA antibodies (Western blot, Panel B). Migration position of the full length Erb-B2-TEV-HA and its clipped form is shown by arrows and position of immunoglobulin heavy and light chains indicated on the right of Western blot panel. Migration of protein markers is shown on the left of each panel.

TEV-mediated cleavage of Erb-B2 mutants in detergent extracts of membranes

Effective cleavage of Erb-B2 mutants by TEV protease is an essential but not sufficient requirement for usage of the proteolysis as purification step. We have tested applicability of TEV proteolysis for generation of purified extracellular Erb-B2 fragment and found out that in spite of cleavage, extracellular N-terminal region of Erb-B2 stays associated with the rest of the molecules and does not become soluble when cleaved on either beads or on cell membranes (data not shown). This finding prevented usage of TEV proteolysis for purification of Erb-B2 fragment. As an alternative approach, we used standard purification procedure based on two steps: metal affinity chromatography on immobilized Co^{2+} ions followed by immunoprecipitation using anti-HA antibodies.

Protein purification/aptamer selection procedure

Alternative purification procedure is schematically depicted on Figure 14. In this approach, 0.5% Triton X-100 extract of the cell membranes prepared from stable cell line expressing Erb-B2-TEV-HA was incubated approximately with 10^{16} molecules of chemical combinatorial library. We used library with 40-base variable region flanked with 22 – 25-base invariant regions for PCR amplification. Exposing entire aptamer library to unpurified extract provided conditions under which only highly specific aptamers bind Erb-B2. Unlike the case,

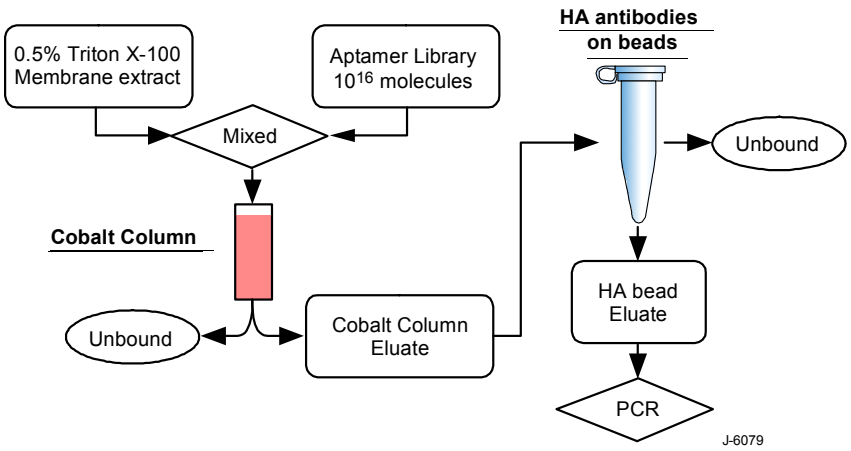

Figure 14. Schematic diagram of the aptamer isolation procedure. Membrane extract was mixed with synthetic combinatorial aptamer library (approximately 10^{16} molecules) or pools of amplified aptamers after each selection step. The mixture was loaded on Co^{2+}-column which was extensively washed to remove unbound proteins and aptamers. Bound material was eluted with imidazole and contained protein/aptamer complexes and minor amounts of free aptamers. The eluate was further used for immunoprecipitation by anti-HA antibodies. Eluate from HA-antibodies contains HA-tagged Erb-B2 mutant with aptamers associated with it. The length of the entire procedure was 18-20 hours which was presumably sufficient for dissociation of weakly bound aptamers.

when purified protein is exposed to the aptamer library, mixing the same aptamer library with a variety of proteins provides and opportunity for aptamers with low affinity to Erb-B2 to bind to other cellular proteins thus reducing overall non-specific binding to Erb-B2. This approach could be viewed as combining a library of aptamers with a library of protein epitopes as opposed to a situation when all aptamers from a library compete for epitopes of a single protein molecule. This approach increase selection pressure and potentially should provide more stringent selection conditions.

After incubation with the aptamer library, protein extract was loaded on Co^{2+}-column followed by extensive wash to remove unbound aptamers and proteins and then bound proteins were eluted by imidazole. This step was not particularly efficient for Erb-B2 purification because substantial amounts of other proteins co-purified with Erb-B2 mutant at this step (Figure 15, Put On lane). However, this column was necessary for removal of unbound aptamers prior to immunoprecipitation to prevent exposure of aptamer library to the immobilized antibodies and agarose beads. Eluted proteins along with aptamer bound to them were further used for immunoprecipitation with anti-HA antibodies.

Co^{2+}-column eluate consisting of protein/aptamer complexes was incubated with anti-HA antibodies immobilized on agarose beads. Two different HA antibody conjugates were tested to identify the one providing better purification (Figure 15, F7 and HA). Majority of proteins did not bind to either of HA conjugate preparation (compare w/out F7 and w/out HA with Co^{2+}-column eluate in Put On lane, Figure 15). After extensive washed, bound material was eluted with low pH Elution Buffer (Pierce). We found that low pH elution from F7 (Santa Cruz)

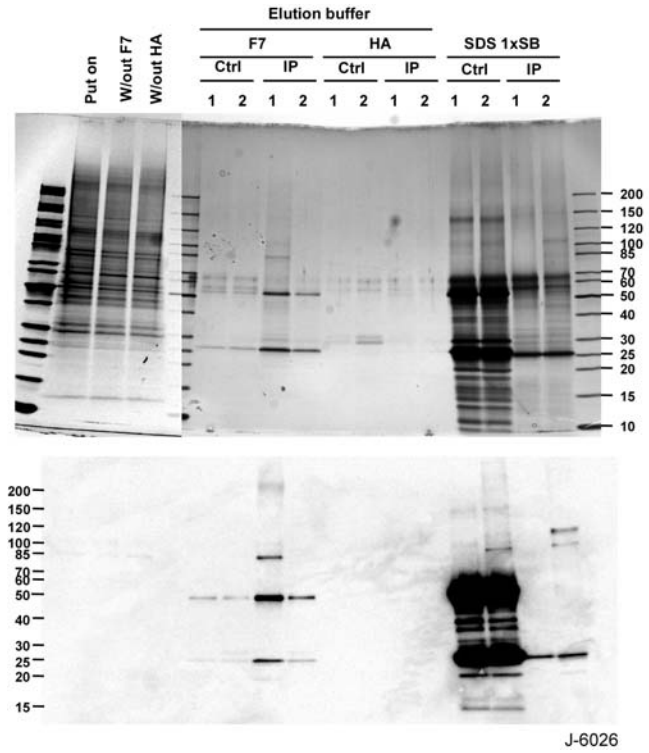

Figure 14. Immunoprecipitation of Erb-B2-TEV-HA complexes with two preparations of HA antibodies. Protein/aptamer complexes eluted from Co^{2+}-column (Put On lane) were incubated with immobilized anti-HA either clone F7, Santa Cruz or HA, Pierce (panels F7 and HA respectively). Lanes 'w/out F7' and 'w/out HA' depict material unbound to F7 or HA resins respectively. Elution was carried out by low pH elution buffer (Pierce) in two fractions: 1 and 2. After the elution, the beads were further extracted by SDS-PAGE loading buffer (SDS 1xSB panel). Top panel shows silver stain and the bottom panel shows Western blot with anti-HA antibodies.

immunoprecipitation resulted in detachment of Erb-B2-TEV-HA in the amounts detectable by silver stain (Figure 14, lanes 1 and 2 in IP F7 panel) in the specific IP but not in the control IP (Figure 15, lanes 1 and 2 Ctrl F7 panel). Elution from HA-agarose (Pierce) resulted in no detectable amounts of the Erb-B2-TEV-HA (Figure 15, HA panel). Additional elution by SDS-PAGE loading buffer mostly resulted in removal of poorly cross-linked immunoglobulins but not in extra yield of the protein of interest (Figure 15, SDS 1xSB panel).

This experiment identified F7 HA-resin as the most suitable for Erb-B2 purification procedure. From this experiment we also concluded that purity of Erb-B2 achieved as the result of two purification steps, Co^{2+}-column and immunoprecipitation with F7 HA-agarose, is suitable for aptamer selection process. This procedure was adopted as a standard and 4 more selection cycles were carried out using combination of Co^{2+}-column and F7 HA-agarose (data not shown).

Aptamers isolated after each HA immunoprecipitation were amplified by PCR for the next selection round. In a standard preparative reaction of 1 ml, we usually produced $10^{13} – 10^{14}$ aptamer molecules. We used biotinylated reverse primer for efficient capturing of PCR products

on streptavidin-conjugated Dynabeads (Invitrogen). Isolated biotinylated double stranded PCR products were extensively washed and 'plus' strand was eluted by mild alkali denaturation. The amount of isolated ssDNA was measured at each cycle by qPCR.

Cloning of isolated aptamers

After four subsequent selection rounds, isolated aptamers were cloned into pCRII vector using T/A-TOPO cloning kit (Invitrogen). Ninety six colonies were picked, expanded and used for DNA isolation for sequencing. About 90 sequencing reaction were successful and ~60 clones contained aptamer inserts. Sequence alignment using Sequencher 4.7 software identified a single group of 18 sequences displaying substantial homology (Figure 16).

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ERB_H05   GCGTCGAAAAAGGTCAGAGCACACG-CATAT-CAGCAACACA--
ERB_F07   GCATCGACACTGGTCAGAGCACAGGCCATCT-CAG-A-CTCACT
ERB_C03   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-ACCTCACT
ERB_C09   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-ACTTCACT
ERB_B04   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-ACTCCACT
ERB_E08   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_E12   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_D09   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_B09   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_B08   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_G12   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_F11   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_G01   GCATCGACACTGGTCAGAGCACAGG-CATCTCCAG-A-CTCACT
ERB_A03   GCATCGACACTGGTCAGAGCACAGG-CATCTCCAG-A-CTCACT
ERB_D08   GCATCGACACTGGTCAGAGCAC-GG-CATCT-CAG-A-CTCACT
ERB_B06   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_F01   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
ERB_F02   GCATCGACACTGGTCAGAGCACAGG-CATCT-CAG-A-CTCACT
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Figure 15. Sequence alignment of aptamers isolated after 4 rounds of selection on Erb-B2 mutant. Only the variable core of 40 bases is shown and the invariant arms used for PCR amplification are removed. Non-identical bases are highlighted in yellow.

The sequence homology suggests that these aptamers will have similar binding properties. However, it is essential to demonstrate whether these aptamer molecules bind to Erb-B2 protein. A binding assay would be an essential step to demonstrate their specificity. However, due to lack time and funds under the current program, this task will be accomplished if further follow up funding is obtained.

KEY RESEARCH ACCOMPLISHMENTS:

- Generation of series of expression constructs encoding for native extracellular and transmembrane domains of human Erb-B2 fused with epitope and purification tags
- Generation of stable cell lines expressing Erb-B2 fusion proteins
- Characterization of stable cell lines expressing Erb-B2 fusion proteins and demonstration of their proper membrane targeting
- Developing a modified aptamer selection procedure based on previously reported SELEX protocol
- Isolation of a group of candidate aptamers and identification their sequence

REPORTABLE OUTCOMES:

Abstract submitted to The Fifth Era of Hope Meeting that will be held on June 25—June 28, 2008, at the Baltimore Convention Center in Baltimore, Maryland.

CONCLUSION:

In course of the research effort supported by fiscal year 2005 Concept Award (Log Number BC053062), we have made significant progress in advancing aptamer technology for detection of breast cancer. Valuable reagents including expression constructs and stable cell lines were generated providing scientific and technological basis for further development of this important project, creation of the next generation targeted contrast agents for MRI. We have selected aptamers against human Erb-B2 and demonstrated enrichment of aptamer pools after four selection rounds. Affinity and specificity of isolated aptamers is yet to be determined.

Isolation of protein-specific aptamers is a highly complex process. During the course of the study, we have developed an expertise and generated reagents essential for further research efforts. Identification of candidate aptamers is very important and is an essential step in generation targeted contrast agents. As the result of this research, we are ready to take this project to the next level of characterization of the selected aptamers and testing them in cell- and tissue-based experimental systems. This research will be continued at Physical Sciences Inc., contingent of future external funding.

All data generated in the course of this research will be shared with scientific community at The Fifth Era of Hope Meeting that will be held on June 25—June 28, 2008, at the Baltimore Convention Center in Baltimore, Maryland.

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APPENDICES: None.

SUPPORTING DATA: None.